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Crystalline Cholesterol: The Material and Its Assembly Lines

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Abstract

Cholesterol is an essential component of animal cell membranes because it influences and controls cell membrane fluidity. Cholesterol is also responsible for the most frequent lethal pathologies in developed countries because of its intimate association with atherosclerotic plaques, the rupture of which may cause heart attacks or strokes. The question is under which conditions cholesterol activity manifests itself, whether in physiology or in pathology. The answer is complex, and there is probably not one certain answer. This review article has its foundations in abundant published knowledge and evidence, but it cannot possibly be comprehensive, because the extent of cholesterol's involvement in chemistry, biology, biophysics, and medicine is so vast that we cannot embrace it all. We review cholesterol as a molecule and in its various crystalline polymorphs. We then examine cholesterol assembly pathways and, finally, cholesterol in biology and in pathology. We propose that cholesterol activity depends on its assembly states in cholesterol crystals or with other lipids in the form of more-or-less organized crystalline domains. In other words, we analyze cholesterol material properties because the assembly state of the cholesterol molecules profoundly affects the properties of the environment in which they reside.

INTRODUCTION

Our instinctive perception of cholesterol inevitably associates it with pathology. Indeed, even the etymology of the word cholesterol, from the Greek chole, meaning bile, and stereos, meaning solid, relates to pathology; that is, cholesterol is the solid that may precipitate from bile. The discovery of cholesterol dates back to approximately the 1760s, when François Poulletier de la Salle, a French chemist, isolated cholesterol crystals from gallstones (never published but cited in References 1–3, among many others). Michel-Eugène Chevreul named the substance cholesterine in 1815, and the name changed to cholesterol after the compound was found to be a secondary alcohol (1, 4).

Felix Boudet, in his 1833 memoir in the *Annales de Chimie et de Physique*, described his discovery of cholesterol in blood. Boudet (5, pp. 340–42) writes,

> Treating with cold alcohol the alcoholic extract of serum, I separated a substance that soon deposited at the bottom of the solution in the form of small crystalline plates very similar to cholesterine. . . . I consulted with M. Chevreul, . . . and following his advice I performed comparative assays between the putative cholesterine isolated from human blood and samples of pure cholesterine extracted from biliary stones . . . . In the previous experiments, the material from blood differentiated from that from biliary stones because of its flaky state and the total absence of crystalline shine, apparently because of the presence of mixed phospholipids. . . . Notwithstanding some differences, I limit myself to signal the existence of cholesterine in blood as extremely probable, although it still needs confirmation on a larger scale.

These early testimonies refer to cholesterol as a material, present in not only pathological but also physiological conditions, distinguishable from other components of blood because of the crystals it forms. Boudet and Chevreul also discovered that cholesterol often appears in conjunction with phospholipids, specifically in the brain, and that the characteristics of cholesterol are not so different from those of the phospholipids that it likes to mix with. These initial observations encompass the most important characteristics of cholesterol as a material as well as the characteristics that establish its functions and allow it to fulfill them.

Little did Boudet know that he had discovered the most abundant animal sterol in blood plasma and body tissues, especially in the liver and brain. To stress the importance of cholesterol and its complexity, suffice it to mention here that Konrad Bloch and Feodor Lynen shared the Nobel Prize in Medicine in 1964 for their discovery of how cholesterol develops from acetic acid via a complex sequence of 36 biochemical reactions (6, 7).

In the laboratory, pure cholesterol appears mainly as a crystalline material. The small, transparent, prismatic crystals deposited from supersaturated acetone solutions do not differ from many other crystalline materials that we encounter in the lab. Together with phospholipids, however, cholesterol changes the properties of the medium with which it interacts, creating a new material with different properties.

When cholesterol deposits pathologically, the material transitions between different crystalline forms with different materials characteristics. In atherosclerotic plaques or in gallstones, the final aggregated, plate-like crystals give rise to materials with properties that must be addressed when treating these diseases. Considering the above, we discuss cholesterol as a material at the borderline between physiology and pathology.

CHOLESTEROL: THE MOLECULE

Cholesterol is a relatively large molecule with the formula $C_{27}H_{46}O$, consisting of three alicyclic six-membered rings and one five-membered ring fused together (the typical backbone structure

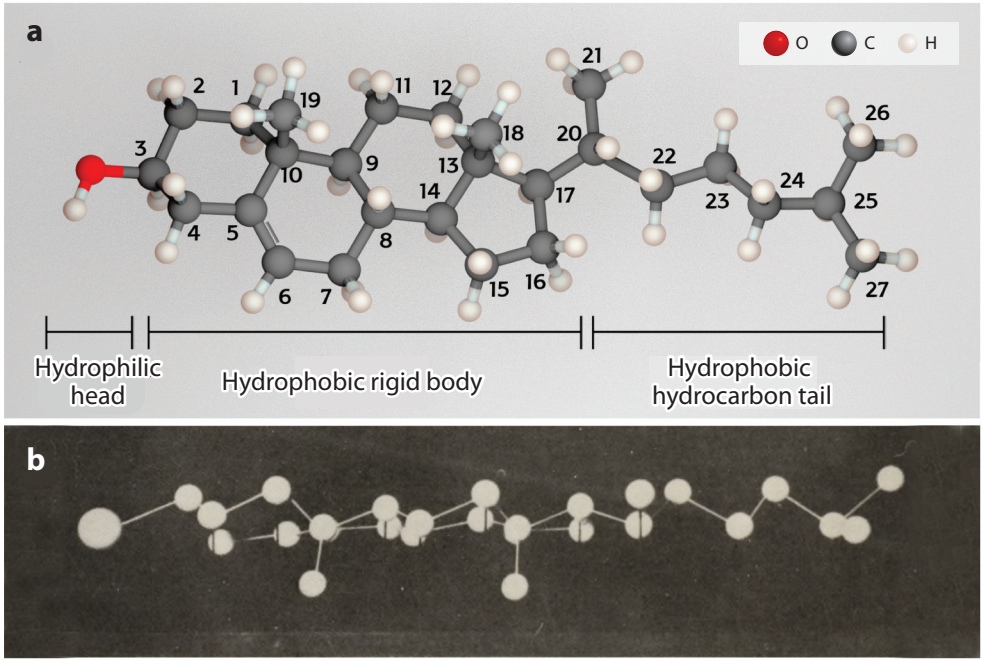

Figure 1

(*a*) Cholesterol's structural formula. The molecule has eight chiral centers at C-3, C-8, C-9, C-10, C-13, C-14, C-17, and C-20, such that the complete formula is (3*S*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-10,13-dimethyl-17-[(2*R*)-6-methylheptan-2-yl]-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol. (*b*) Model of the cholesterol molecule as Dorothy Crowfoot Hodgkin derived it from the cholesteryl iodide structure. Panel *b* reproduced with permission from Reference 8.

of sterols) and an additional aliphatic tail, forming a rigid, hydrophobic molecule (**Figure 1*a***). The hydroxyl group in position 3 of the molecule is the only hydrophilic moiety. Due to its hydrophobic nature, the cholesterol molecule is practically insoluble in water (its solubility in aqueous solutions is 67 nM) (9) and thus needs to dissolve in hydrophobic environments. Cholesterol achieves this in biological systems through incorporation into lipid membranes, in complex formations with bile salts in the liver, or with lipoproteins in blood (10, 11).

Carlisle & Crowfoot (8) (later Hodgkin) determined the complex structure of the sterol skeleton in cholesteryl iodide in 1942 and published it in 1945 (**Figure 1*b***). They wrote (p. 64),

> The present X-ray analysis of cholesteryl iodide (I) is an attempt to determine the detailed structure of the sterol skeleton. . . . It is the natural continuation of the crystallographic measurements on sterols begun by J. D. Bernal in 1932 and illustrates some of the general problems involved in the . . . determination of the chemical structure of complex organic molecules. . . . There still remain problems in detail, particularly of stereochemistry, which need further investigation.

Indeed, the cholesterol molecule has a very complex stereochemistry, with eight chiral centers (**Figure 1*a***). This means that the cholesterol molecule has 2^8 possible stereoisomers, suggesting that theoretically there are 256 different molecules with the same formula and backbone as cholesterol but presumably with vastly different properties (12). Of these 256 possible stereoisomers, virtually only one occurs in nature (epicholesterol exists but is extremely rare). It is interesting to consider how the chirality and stereochemistry of cholesterol may influence its interactions with the environment. In cholesterol, the chiral centers are distributed so that by and large the molecule

assumes a 17-Å long, 6-Å wide, remarkably linear shape with a flat asymmetric structure defined by a planar side and a rough side, where the methyl moieties emerge (**Figure 1*b***) (13, 14). This three-dimensional (3D), elongated, quasi-cylindrical shape makes cholesterol molecules particularly apt to intercalate between the hydrophobic phospholipid chains in cell membranes. The hydrophobic region intercalates within the fatty-acid chains of the lipids in the cell membrane, whereas the hydroxyl group points toward the water molecules surrounding the membrane. Phospholipids in particular have large hydrophilic head groups relative to their slim hydrophobic bodies, composed of aliphatic chains, whereas cholesterol has a small hydrophilic head and a large, rigid hydrophobic body (**Figure 1*a***). Because of its hydrophobic nature, its stereochemistry, and its volume and geometry, the cholesterol molecule interacts favorably with saturated glycerophospholipids and especially with sphingolipids in the cell plasma membrane and other cellular membranes. This property makes cholesterol an important factor for regulating cell membrane fluidity, stabilizing the membranes, and preventing phospholipid crystallization (7). It is evident that different stereoisomers with altered geometries would interact differently with cell membranes. There is also evidence that cholesterol and its enantiomer, ent-cholesterol, influence the properties of lipid membranes differently (15).

To fulfill its functions in the regulation of membrane properties, cholesterol molecules must reach all the cells in the organism (16). For this purpose, cholesterol molecules are transported in the blood by low-density lipoproteins (LDLs) in the form of cholesterol and cholesterol ester. Excess cholesterol may be stored in the cell in lipid droplets, which are predominantly composed of cholesterol and its ester, or can be removed from the cells back into the blood flow by high-density lipoproteins (HDLs). Excess cholesterol, however, can crystallize, forming deposits in the artery walls or stones in the gall bladder. Once deposited, cholesterol crystals are very difficult to dissolve in the aqueous environment predominating inside the organism and thus give rise to pathological conditions that may be life threatening (17).

CRYSTALS AND CRYSTAL STRUCTURES

Five structures of cholesterol and hydrated cholesterol crystals have been determined so far. The best-known cholesterol crystal structure is the triclinic cholesterol monohydrate determined by Craven (18) in 1976, more than 30 years after Dorothy Hodgkin's determination of the X-ray structure of cholesteryl iodide (**Figure 2*a***). The relatively late determination of the structure was due to the complexity not only of the molecule but also of the crystal structure: The space group is triclinic P1, with eight independent molecules/unit cell ($a = 12.39$ Å, $b = 12.41$ Å, $c = 34.36$ Å, $\alpha = 91.9°$, $\beta = 98.1°$, $\gamma = 100.8°$). The unit cell thus contains 616 atoms, making it the size of a small protein. In 1977, the determination of the cholesterol monohydrate triclinic structure was followed by the determination of the anhydrous cholesterol structure, which is also triclinic P1 with eight molecules/unit cell ($a = 14.17$ Å, $b = 34.20$ Å, $c = 10.48$ Å, $\alpha = 94.64°$, $\beta = 90.67°$, and $\gamma = 96.32°$) (**Figure 2*c***) (19). The anhydrous cholesterol crystals were obtained from acetone in the absence of water.

In 2005, Solomonov et al. (20) determined the structure of a monoclinic polymorph of cholesterol monohydrate, which forms from compressed monolayers of cholesterol transforming into multilayers at the air–water interface (**Figure 2*b***). The monoclinic form has eight molecules/unit cell ($a = 10.15$ Å, $b = 7.57$ Å, $c = 68.20$ Å, $\alpha = 90.0°$, $\beta = 94.8°$, $\gamma = 90.0°$), but because of the higher symmetry, there are two molecules/asymmetric unit. Talmon, Konikoff & colleagues (21) detected the same crystal structure in cholesterol crystals nucleating from bile acid mixtures.

Next, we analyze the similarities and differences between the three structures, trying to derive conclusions about their growth patterns and stabilities. In all three structures, the cholesterol

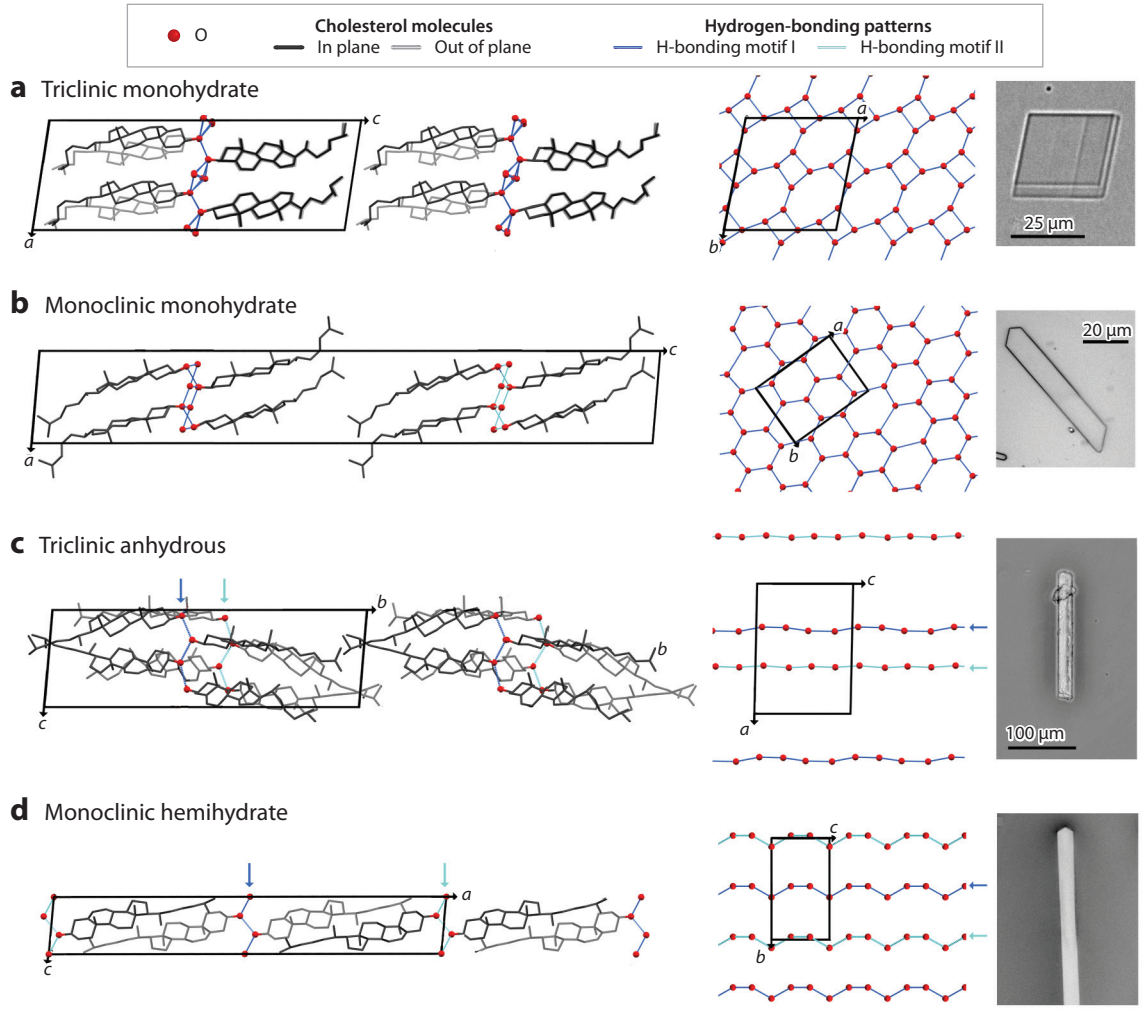

Figure 2

Packing arrangement of cholesterol in four out of five known crystal structures (18–20, 22). The far right of each panel shows images of the respective crystals. (*a*) Triclinic cholesterol monohydrate packing arrangement viewed along the *b* axis (*left*); two unit cells, corresponding to two bilayers, are represented along the *c* axis, whereas only half a unit cell is represented along the *b* axis. On the right is a view along the *c* axis of the hydrogen-bonding pattern. (*b*) Monoclinic cholesterol monohydrate viewed along the *b* axis, and the hydrogen-bonding pattern viewed along the *c*-axis. Note that there are two hydrogen-bonding patterns in the two bilayers, only one of which is shown. (*c*) Triclinic cholesterol anhydrous viewed along the *a* axis and the hydrogen-bonding pattern viewed along the *b* axis. Note that the two line motifs of hydrogen bonds are on two different planes. (*d*) Monoclinic cholesterol hemihydrate. The cholesterol molecules pack in bilayers, but within each bilayer, neighboring molecules have opposite orientations, in contrast to the previous structures. Two line motifs of hydrogen-bonded molecules form at two different levels. The hemihydrate crystals are 2.3 mm long and 0.2 mm wide on average. The crystal image in panel *d* was kindly provided by M.B Al-Handawi and P. Naumov from NYU Abu Dhabi.

molecules pack in bilayers (**Figure 2**). Layers of cholesterol molecules interacting through hydrogen bonds between their hydrophilic heads (and with intercalated water molecules, when present) alternate with layers of molecules interacting through their hydrophobic tails. In general, all three crystal structures, and the two triclinic ones in particular, are complicated because of the tension between the formation of hydrogen bonds at the hydrophilic interface and the establishment of

viable hydrophobic contacts between the backbones and side chains. A compromise must be reached, which translates in the triclinic structures into a lack of symmetry and the complex spatial relations of the rigid backbones. Because of constraints arising from the rigid cholesterol alicyclic ring structure, in the anhydrous cholesterol structure, there cannot be hydrogen bonds between molecules within the same layer. Hydrogen bonds are therefore established between molecules in two juxtaposed layers, and the hydrogen-bonding pattern is distorted into two different one-dimensional chains at two different levels (**Figure 2*c***). In the monohydrate structures, the intercalated water molecules relieve the spatial constraints. The hydrogen bonding networks can develop into two-dimensional (2D) planar patterns spanning the hydrophilic interface, and for this reason, the monohydrate structures are favored in the presence of even minimal amounts of water (**Figure 2*a*,*b***).

The monoclinic cholesterol monohydrate structure is more ordered and has higher symmetry than either triclinic structure. The more regular structure is visible in the ordered packing of the hydrophilic moieties, which form a flat 2D pattern of hydrogen bonds (5 Å × 7.5 Å), close to that of hexagonal ice (23). The contacts between the alicyclic rings within the layer are also complementary, with the flat sides of adjacent molecules facing each other in one pair, alternating with molecular pairs with interleaved methyl groups. The monoclinic polymorph is stable in bilayer domains, and so far, the reader might believe that monoclinic cholesterol monohydrate crystals should be more stable than the triclinic polymorph, but this is not true. Triclinic cholesterol monohydrate is the most stable polymorph, the polymorph that grows in vitro from water or from organic solvents in the presence of minor amounts of water, and the polymorph that is detected in atherosclerotic plaques and gallstones (24, 25). The triclinic polymorph has higher density than the monoclinic one, and, more importantly, the monoclinic polymorph transforms into the triclinic one as molecular layers assemble in the transition from bilayer to multilayer. We attribute this phenomenon to the hydrophobic contacts between bilayers, which are much more extensive in the triclinic structure of cholesterol monohydrate than in the monoclinic structure. Indeed, density-functional theory calculations show that at 0 K, the difference in stability between the two polymorphic structures is minor. At higher temperatures, the triclinic polymorph becomes more stable by virtue of entropy, that is, because of the lack of molecular symmetry constraints in the crystal structure (23).

Triclinic cholesterol monohydrate crystals grow from acetone as bulky prisms, but as the water percentage increases, they develop into thinner rhomboid plates, growing much slower in the direction of the crystallographic *c* axis, perpendicular to the molecular bilayers (**Figure 2*a***) (26, 27). This phenomenon is attributable to the intercalation of excess water molecules within the hydrophilic layer, inhibiting the attachment of new *ab* cholesterol layers in the *c* direction. This type of plate appears from crystallizations in vitro, in gallstones, and in atherosclerotic plaques. The telltale angle of 101° in the plate ensues from the unit cell angle γ between the *a* and *b* axes, which are parallel to the plate sides. The *a* and *b* axes are almost equivalent in dimensions and in molecular interactions such that the plates tend to be almost equilateral.

In 1992, Konikoff et al. (28) published a paper on "Filamentous, Helical, and Tubular Microstructures During Cholesterol Crystallization from Bile." These microstructures were followed in time as they transformed: filaments followed by helical wires grew laterally to form helical ribbons and then tubules, which fed the conventional cholesterol rhomboid plates that grew from them.

Helical ribbons were established as metastable intermediates in the process of cholesterol crystallization in bile and in synthetic analogs of bile composed of bile acids, phosphatidylcholine, and cholesterol (29). The helical and tubular microstructures were initially believed to consist of a kind of liquid crystalline mixture of the three types of chiral molecules: bile acids, phosphatidylcholine,

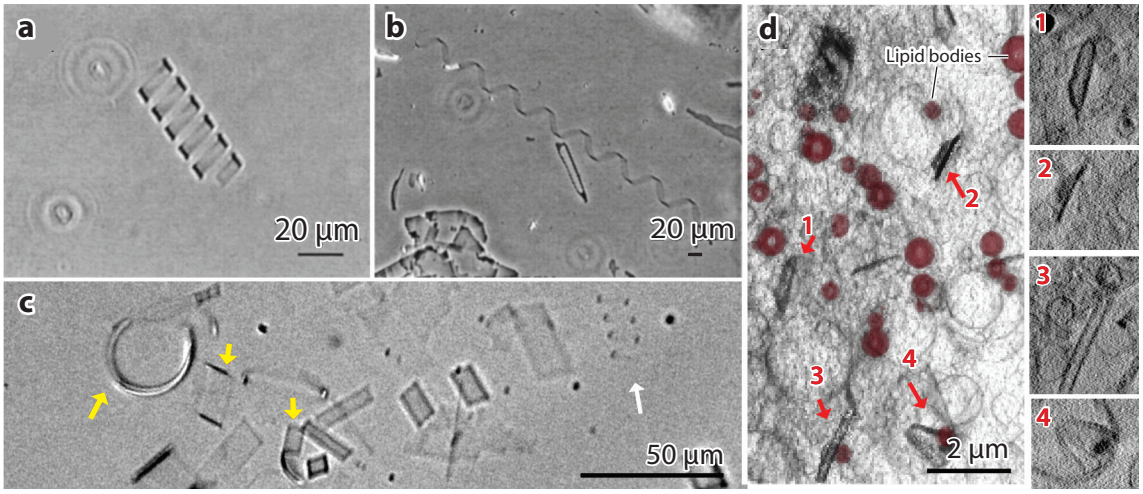

Figure 3

Helical and tubular structures from cholesterol crystallization in models of bile, in macrophage cells, and from bilayers of cholesterol mixed with phospholipids. (*a*,*b*) Cholesterol helices with 11° and 54° pitch, respectively, from cholesterol crystallization in model bile. Images in panels *a* and *b* reproduced with permission from Reference 30; copyright 1999 National Academy of Sciences. (*c*) Cholesterol helix (*white arrow*), tubules, and sheets (*yellow arrows*) from cholesterol crystallization from bilayers of cholesterol and dipalmitoyl-phosphatidyl-choline. (*d*) Volume representation of a cryo-soft X-ray tomograph of tubular cholesterol crystals (*red arrows*) grown by a macrophage cell in intimate contact with cell membranes (*gray*). Inserts 1, 2, 3, and 4 are slices through the tubular crystals indicated by the red arrows.

and cholesterol. It was discovered later that similar helical and tubular structures also form from different mixtures containing a nonionic detergent, a fatty acid, and steroids other than cholesterol. The helical structures develop progressively in time from helices with a high pitch of 54° to helices with a low pitch of 11° (**Figure 3**) (30–32). These are two different, well-defined species with different structures that do not transform directly into one another. In 2007, Benedek and colleagues (32) measured well-defined diffraction patterns from stretched, low-pitch helical ribbons and from them determined a new cholesterol crystal structure. This structure well matches the triclinic monohydrate structure, but the length of the unit cell perpendicular to the cholesterol layers is tripled, that is, the unit cell dimensions are $a = (12.1 \pm 0.4)$ Å, $b = (12.1 \pm 0.4)$ Å, $c = (102 \pm 5)$ Å, $\alpha = (90 \pm 3)°$, $\beta = (97 \pm 3)°$, and $\gamma = (102 \pm 2)°$. Benedek and colleagues suggested that slippage of layers might occur to compensate for the curvature in the helical ribbon; the need to compensate for the difference between the concave and convex part of the helical ribbon could result in the formation of a superlattice.

We note that the high-pitch structure may be different from that determined by Benedek and colleagues (32) for the low-pitch helices, and we have some reason to believe that the high-pitch helices may consist of very thin crystals of cholesterol monohydrate in the monoclinic structure. This possibility arises from the detection, by electron diffraction, of monoclinic crystals nucleating from bile acid mixtures (21). In addition, helical crystals with an estimated pitch of 60° also form in macrophage cells supplemented with cholesterol and from bilayers of cholesterol mixed with phospholipids (**Figure 3*c*,*d***) (33, 34). In the latter two cases, from cell cultures and from hydrated bilayers, and in all morphologies, tubular, helical, or flat plates, the crystals have the structure of the monoclinic polymorph. The crystals develop along the *b* direction, whereas along the *c* axis, where the bilayers attach to one another, the crystals are only a few bilayers thick, within the range of a few tens of nanometers. With the addition of new bilayers, the helices may become elongated

rods and may undergo phase transition to the triclinic form or may stretch out and fold back, transitioning to the triclinic crystal plate morphology and structure (21, 34). Interestingly, Konikoff & Carey (29) observed that needle-like crystals developed from mixtures of bile acids and cholesterol without phosphatidylcholine, whereas plates develop directly in the absence of bile acids. Furthermore, an examination of human samples revealed that "needle-like cholesterol crystals predominated in [the bile] of most patients without gallstones, whereas plate-like and dot-like crystals were more common in patients with gallstones. All three crystal types were seen in most patients" (35, p. 364).

The driving force behind the formation of the helical crystals is not clear; in the bile systems, modeling of the helices applied the formalism of elastic free energy to fluid amphiphilic chiral bilayers, achieving a match to the geometrical features observed experimentally. Various interpretations that could be individually proven or disproved are conceivable for the formation of the helices, including topological defects such as screw dislocations (36–38), boundary effects, or spontaneous curvature induced by molecular tilt (32, 39). Of particular interest is the possible effect of molecular chirality in breaking symmetry, thus leading to helix formation (40). In an extensive statistical determination, Benedek and colleagues (30, 31) report that right-handed helices are predominant in the formation of cholesterol helices from artificial bile, but 5% of the helices are left-handed, which occurs only in the high-pitch helices. It would be interesting to investigate the feasibility of using such helices and tubules of cholesterol monohydrate as carriers for drugs in the blood or, as has been suggested, as micro-mechanical devices in a range of applications from biology to electronics (41, 42).

The most recent cholesterol crystal structure determined is cholesterol hemihydrate (**Figure 2*d***) (22). The crystal structure of the rod-like hemihydrate crystals obtained from hexanol or octanol solvates is monoclinic C2, with one molecule of cholesterol and half a molecule of water in the asymmetric unit. The cholesterol molecules pack in bilayers, with the hydrophilic heads of translationally related molecules aligned in columns in the direction of the *c* axis, but in contrast to the previous structures, within the bilayer along the *a* and *b* axes, neighboring molecules have opposite orientation. For this reason, there is no extended 2D network of hydrogen bonds between the hydroxyl groups of the cholesterol molecules and the water molecules. Rather, each of the two hydroxyl groups form with one interleaved water molecule the repeating unit of hydrogen-bonded chains along *c*, in agreement with the hemihydrate stoichiometry.

Thus in total, there are five crystal structures for cholesterol: one anhydrous, three monohydrate, and one hemihydrate. There are three main observed morphologies: rhomboid plates, rods, and helical or cylindrical ribbons. In atherosclerotic plaques, both rhomboid plates and rods are found, but according to X-ray determinations (43), the only crystal structure present is the stable triclinic cholesterol monohydrate structure. Because this is the most stable structure of cholesterol, it is conceivable that even though other polymorphs are deposited, these may transform into the most stable polymorph with time.

Anhydrous cholesterol, the monoclinic cholesterol monohydrate, and cholesterol hemihydrate all form elongated crystals. This is a subject of interest, because according to one current hypothesis, rod-like crystals of cholesterol may perforate the cell membrane, causing mechanical damage and subsequent cell death, giving rise to inflammation. According to similar mechanical damage hypotheses, cholesterol crystals may perforate the fibrous cap of a plaque, causing plaque rupture and subsequent formation of thrombi (22, 44–47). In the hemihydrate case, the crystals grow as pointed needles along the *c* axis, where the intermolecular interactions are highest. Performing tensile mechanical measurements on model membranes, Naumov and colleagues (22) concluded that the crystals are indeed sufficiently rigid to rupture the cap of an atherosclerotic plaque.

CHOLESTEROL AGGREGATION STATES

When cholesterol crystallizes in an aqueous biological environment, in many respects, this is the end of the story, because cholesterol crystals are extremely difficult to dissolve in water. However, if cholesterol is so difficult to dissolve in water, how did cholesterol get there in the first place, and how did it crystallize? In this section, we report on cholesterol assembly pathways and states.

Because of their amphiphilic character, cholesterol molecules tend to partition at the boundary between an aqueous phase and a nonpolar aprotic phase. However, the interaction of the large hydrophobic backbone with the environment is dominant over the ability of the hydroxyl group to participate in hydrogen bonding with polar solvents. Practically, the molecule behaves as hydrophobic: It completely disperses in nonpolar solvents, such as benzene, whereas it aggregates at very low concentrations in protic polar solvents, such as water (13, 14). Cholesterol tends to aggregate in water by lateral packing, forming double-layer structures as it does in the crystalline state. Because of the stronger backbone-to-backbone interactions, growth within the layers is much faster relative to growth across different layers, and micelles of different geometry may result (**Figure 4*a***). The critical concentration for cholesterol micellization in water is 25–40 nM at 25°C (14).

The forces that stabilize the micelle aggregations arise in part from hydrophobic repulsion by water molecules. Cholesterol molecules show anomalous hydrophobicity values, which deviate from the linear relation between hydrophobicity and the hydrocarbon surface areas of alkanes, alcohols, and carboxylic acids (48–50). The hydrophobic surface area per molecule estimated for the removal of a cholesterol dimer from the aqueous phase is lower than that estimated for a single molecule (51). The free energy of micellization is therefore higher than would have been predicted from the intrinsic hydrophobicity of cholesterol alone. The additional favorable energy presumably arises from structural considerations, where extra-attractive forces between molecules favor self-association through packing of cholesterol backbones and water molecules (50).

In aqueous biological environments, cholesterol favors mixed surfactant-like media, such as lipid bilayer membranes or mixed micelles and vesicles with biliary acids. When cholesterol is supersaturated, it tends to segregate from these mixed lipid environments, forming crystalline domains (52–54). Grazing incidence diffraction studies on model bilayer membranes show that above saturation, cholesterol forms ordered domains with sizes of tens of nanometers (50–80 nm) and with the thickness of a bilayer (3.5 nm). The driving force for the segregation of cholesterol to form distinct domains conceivably depends on the cross-layer interaction, because cholesterol crystalline domains do not form in monolayers (**Figure 4*b***) (55). In fact, in monolayers, cholesterol can be considered as a free rotator that has free motion around its molecular axis (56).

The cholesterol crystalline domains that form in lipid bilayers are 2D cholesterol monohydrate assemblies that pack in a 10×7.5 Å^2 rectangular unit cell with the bilayer structure of the monoclinic polymorph (**Figure 4*c***) (20, 52–54). Interestingly, the stable triclinic polymorph of cholesterol monohydrate crystals was never detected as a bilayer-thick crystal, while the metastable monoclinic polymorph exists as both bilayers and multilayers. The 2D monoclinic crystalline domains can mature and develop into 3D crystals of the monoclinic polymorph with helical, cylindrical, or flat platelet morphology or can transform into the triclinic polymorph at early stages (**Figure 4*e***) (20, 34, 54, 57). Several observations point to the possible effect of the environment from which cholesterol crystals nucleate and grow on the kinetics of growth of one crystal polymorph relative to the other. For example, lipid mixtures composed of sphingomyelin with high cholesterol concentration produce monoclinic cholesterol monohydrate 2D crystalline assemblies (52). When supplied with exogenous cholesterol, macroscopic 3D triclinic crystals form, which are intimately associated with the bilayer (34). In contrast, when exogenous cholesterol is supplied

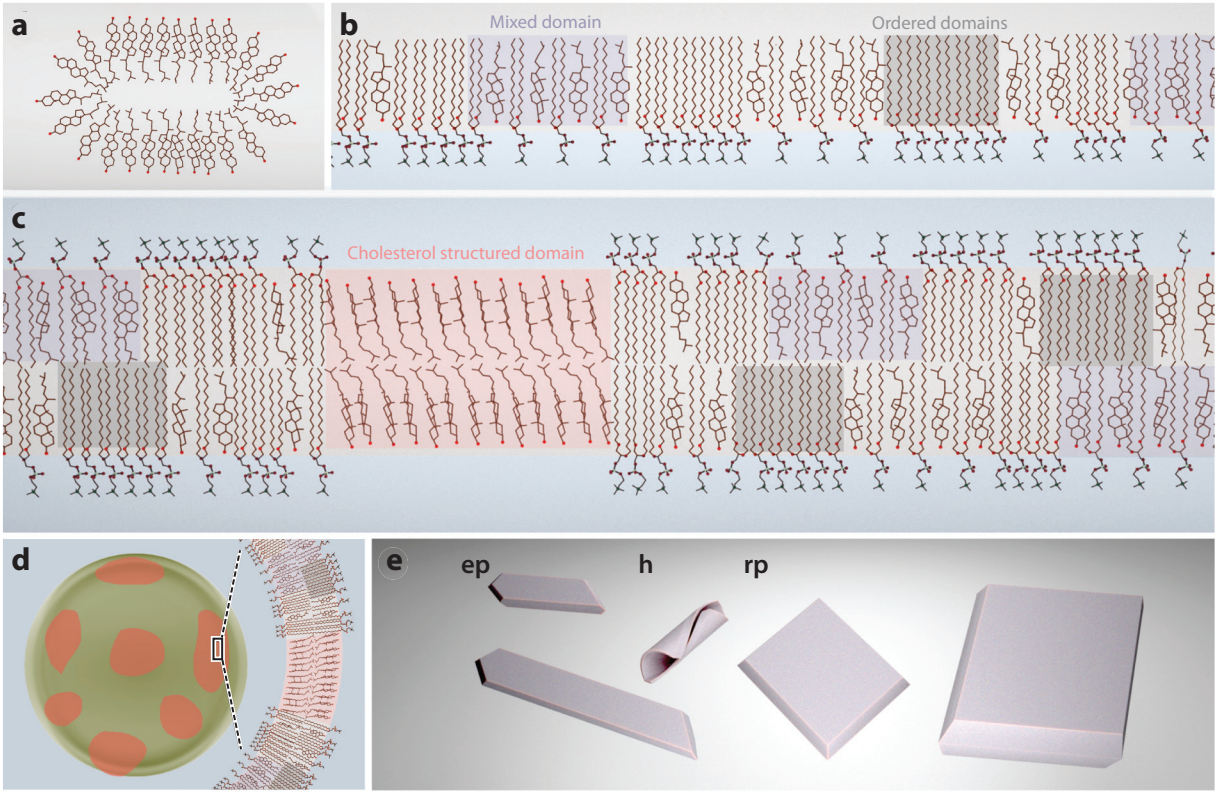

Figure 4

Schematic representation of different cholesterol aggregation states. (*a*) Cholesterol micelle in one possible geometrical configuration. (*b*) Mixed monolayer of cholesterol with phospholipids. Cholesterol does not self-assemble in structured domains but forms ordered mixed domains with saturated phospholipids (*purple*). The saturated phospholipids may form self-assembled ordered domains (*gray*). (*c*) Mixed bilayer of cholesterol with phospholipids. Cholesterol may self-assemble in structured domains of bilayer thickness (*pink*) and form ordered mixed domains with saturated phospholipids with monolayer thickness (*purple*). The saturated phospholipids may form self-assembled ordered domains with monolayer thickness (*gray*). (*d*) Giant vesicle (*green*) with micrometer-size domains (*red*), as canonically observed by fluorescence. It is conceivable that such large domains are actually composed of distinct, ordered nanodomains. The magnified schematic from the ordered red area is as represented in panel *c*. (*e*) Schematics of crystal forms that can emerge from cholesterol crystallization in different environments. From left to right, these include (*ep*) elongated plates and (*h*) helical or tubular crystals from monoclinic cholesterol monohydrate and (*rp*) rhomboid plates from triclinic cholesterol monohydrate.

under similar experimental conditions to bilayers composed of the saturated lipid dipalmitoyl-phosphatidyl-choline (DPPC), the 2D bilayer monoclinic crystals (53) develop, at least in part, into 3D monoclinic crystals (34, 57).

The interplay between the two crystal polymorphs and the possible effect of the environment on their relative stability is still under investigation. Understanding the mechanism of cholesterol aggregation and crystal growth in amphiphilic environments is a key for understanding cholesterol's crystallization pathway in biology and appears to be very relevant for pathology.

To understand the equilibrium of cholesterol states in biology, we must understand the physiological functions that make it essential in animal cells. In addition to other functions that we shall not address here, such as in signaling or hormone synthesis, a fundamental function of cholesterol in cell membranes relates directly to its assembly and material properties. Cholesterol helps to regulate membrane fluidity and plays a role in forming and maintaining cell membranes and

structures (58–60). Key functions depend on the cholesterol ordering effect, that is, its ability to form ordered domains in mixtures with other lipids. Below, we summarize the relevant concepts of a large volume of literature on the subject.

Cholesterol's mode of incorporation in lipid bilayers is, not surprisingly, dependent on how cholesterol can integrate in the hydrophobic lipid region. In mixed cholesterol–lipid systems, the molecules tend to pack laterally to form bilayers where the hydrophobic part of cholesterol is embedded along the lipid hydrocarbon chains. The orientation and location of cholesterol within the bilayer is determined mostly by the length of the lipid hydrocarbon chain. In the most common orientation, the hydroxyl group of cholesterol is oriented toward the water interface. However, when the length of the hydrocarbon chain is shorter than half the length of cholesterol (15 Å), the molecule can orient parallel to the bilayer and locate at the bilayer midplane (61).

The planar and rigid structure of cholesterol limits the possible conformations that the molecule can adopt and dictates the preferred interactions of cholesterol with the chains of other lipids in mixed bilayers. Cholesterol's relative affinity for the different lipids depends on the degree of saturation of the lipid chains (62, 63). In general, cholesterol has stronger interactions with sphingolipids, such as sphingomyelin, and saturated long-chain glycerophospholipids, such as DPPC, relative to unsaturated lipids such as 1-palmitoyl-2-oleoyl-phosphatidylcholine (64, 65). The different affinity of cholesterol for the different types of lipids and lipids' tendency to crystallize by themselves induce nonrandom distribution of cholesterol in mixed lipid membranes.

In the late 1980s, biologists introduced the raft designation to indicate the existence of relatively stable compartments in the cell plasma membrane that are important in regulating biological processes such as protein sorting, signal transduction, and cell adhesion (66, 67). These domains are rich in cholesterol, sphingolipids, and saturated long-chain glycerophospholipids and have distinct structure and kinetics relative to the rest of the cellular membrane (68). The concept of heterogeneous lipid–lipid interactions and of possible compartmentalization resulting from these heterogeneous interactions was not new to chemists and physicists. Modeling of cell plasma membranes in vitro, however, is made extremely difficult by the complexity of the cell membrane components, which can reach up to more than 1,000 lipid species, even without taking into account the proteins. Interestingly, there is only one sterol in animal cell membranes, and this is cholesterol.

Model membrane studies that evaluate cholesterol–lipid interactions are therefore necessarily simplified and often use ternary mixtures composed of cholesterol and a saturated and unsaturated lipid (69, 70). Although chemically simplified mixtures of a few lipid components do not entirely reflect the properties and dynamics of biological membranes, it is possible to characterize the structural parameters of the mixtures across the whole range of concentrations. It is important to stress, however, that the derived structural parameters refer to systems under equilibrium, a situation very far from what occurs in biology. In live cells, membrane recycling as well as membrane fusion and vesicle detachment continuously change the local composition and the organization of the membrane and are part of essential cell functioning. Achieving equilibrium in cells is equivalent to death.

In three-component mixed compositions, depending on the ratios of the lipids, several different phases may coexist, with different regions having different properties. The unsaturated lipid forms with cholesterol a liquid-disordered phase, while the good packing of cholesterol with saturated lipids gives rise to liquid-ordered (*Lo*) phases. At low cholesterol concentrations, cholesterol molecules, in addition to packing with saturated lipids, may form dimers within the layer (71) or tail-to-tail dimers across the layers (72, 73). The ordered lipid–cholesterol phases are mixed crystalline domains that can coexist with disordered phases or segregated crystalline phases of lipids alone. The boundaries of the ordered phases are believed to be populated by a less ordered layer of

lipid molecules that lowers the line tension between different ordered domains and the disordered lipid environments (74).

The exact size and shape of the lipid ordered domains, however, appear to vary with the method of observation and the cholesterol–lipid concentration. The majority of studies have used fluorescence microscopy to visualize ordered phases in giant vesicles (**Figure 4*d***) (75–77), planar systems of supported lipid monolayers (75, 78), and supported lipid bilayers (75) and report the domains to have sizes of several micrometers up to tens of micrometers. X-ray diffraction, nuclear atomic resonance, and atomic force microscopy report on cholesterol-rich domains with sizes ranging between several nanometers to hundreds of nanometers (52–54, 79). Interestingly, the mixed crystalline domains are only one layer thick, that is, they comprise only one bilayer leaflet (**Figure 4**) (55). It is conceivable that a single phase with a size of several micrometers that can be detected by fluorescence is actually composed of distinct ordered nanodomains that cannot be detected by fluorescence due to resolution limitations and possibly because they grow upon intense illumination (80–82).

For the ordered domains to control protein location and activity, it is essential for the cell to be able to control the fraction of the membrane in the *L*o phase. Indeed, cholesterol levels in cells and the distribution of cholesterol in the different membranes within the cell are strictly controlled. The concentration of cholesterol in the endoplasmic reticulum, where it is synthesized, is relatively low. Its concentration progressively increases along the secretory pathway and reaches its highest levels in the plasma membrane, where cholesterol can constitute almost 50% of the lipid composition (83, 84).

The different distribution of cholesterol in membranes causes changes in their physical properties (60), making them less permeable as cholesterol concentration increases and more readily deformable at low cholesterol concentrations (59). The ordering effect that proximity with cholesterol molecules induces on the hydrocarbon chains of neighboring lipids effectively reduces the area occupied by each lipid and therefore increases the packing of the membrane and reduces its permeability (58, 59). Cholesterol can also have the opposite effect when mixed with ordered lipids, as it can incorporate between lipids that have a tendency to pack tightly and inhibit the formation of a packed gel crystalline phase (59), thus locally increasing membrane fluidity. It was demonstrated, however, that when cholesterol is incorporated with unsaturated lipids, it also results in a local stiffening effect on the membrane (85).

It is conceivable that at very high cholesterol concentrations, especially in the plasma membrane or under pathological conditions, pure cholesterol domains may form, even in regions where there should be miscibility with other lipids. These domains, being one bilayer thick, may locally change the mechanical properties of the membrane by stitching together the bilayer leaflets.

Clearly, regulation of the cholesterol levels in membranes is of primary importance. When the balance of cholesterol homeostasis is disrupted, this has a direct effect on membrane function and can lead to several pathological outcomes. One direct consequence is the precipitation of cholesterol as monohydrate crystals when cholesterol reaches saturation (43, 86).

CHOLESTEROL AGGREGATION STATES IN PHYSIOLOGY AND PATHOLOGY

Cholesterol is an integral constituent of membranes, lipoproteins, bile acids, steroid hormones, and more. Its synthesis, esterification, and hydrolysis, as well as its metabolism in general, must be closely monitored.

To ensure a controlled cholesterol concentration in the human body, endogenous and exogenous metabolic pathways must coordinate (87). The liver and extrahepatic tissues synthesize

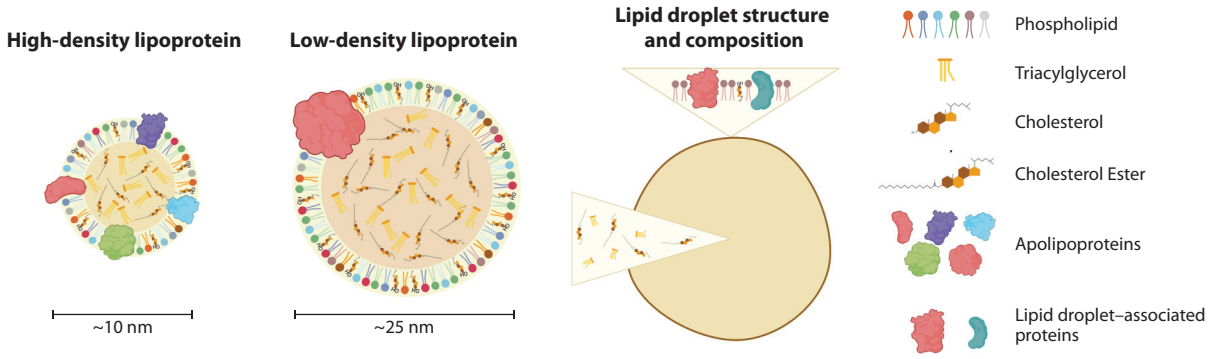

Figure 5

Schematic representation of high-density lipoprotein (HDL) particles, low-density lipoprotein (LDL) particles, and lipid droplets with their characteristic compositions. HDL particles have a diameter of ~10 nm whereas LDL particles have a diameter of ~25 nm. Lipid droplets are much larger and may reach several tens of micrometers in diameter. Figure adapted from images created with BioRender.com.

cholesterol via the endogenous pathway (88). Following its synthesis, cholesterol enters the blood circulation or is secreted into bile. Cholesterol from dietary and biliary sources is absorbed via the exogenous pathway in the intestine and subsequently enters circulation.

To fulfill its many functions, cholesterol must reach cells all over the body. For this purpose, cholesterol traffic is controlled in the blood by specialized lipoproteins. The lipoprotein carriers are classified into four classes, chylomicrons, very low-density lipoprotein, LDL, and HDL, which differ from one another in size, density, lipid composition, and protein composition (16). These complex particles are comprised of a central hydrophobic core of nonpolar lipids such as triglycerides and cholesterol esters, which is surrounded by a monolayer consisting of phospholipids, free cholesterol, and apolipoproteins (**Figure 5**).

The two major lipoproteins associated with cholesterol transport and metabolism are HDLs and LDLs. LDLs, often incorrectly referred to as bad cholesterol, are particles with sizes around 25 nm that transport cholesterol from the liver through the vasculature and distribute it among the various tissues. Upon reaching the cells, LDL particles are taken up and processed and cholesterol becomes available. HDLs, often referred to as good cholesterol, are smaller particles with sizes around 10 nm that absorb cholesterol and carry it back to the liver. Roughly 50% of LDL and 20% of HDL compositions are esterified cholesterol and cholesterol (**Figure 5**) (89, 90).

The dynamic properties of the superficial lipid monolayers mostly depend on the nature of the lipid component. The surface lipids of LDLs are more packed and rigid than those of HDLs due to the presence of a larger percentage of saturated fatty acids in the phospholipids, a higher sphingomyelin-to-glycerolipid ratio, and a higher cholesterol-to-phospholipid ratio (16, 91, 92). Under physiological conditions, the interior of a lipoprotein takes the form of a liquid droplet of neutral lipids. Within an HDL, due to the relatively small volume available, the core of neutral lipids seems not to be organized. The physical state of cholesterol esters and triacylglycerol molecules in the core of LDLs is more complex to determine due to a reversible, broad phase transition at around 30°C (92–94). The core lipids are in a liquid state above the phase transition and in an ordered smectic liquid-crystal phase below it, but the precise temperature of the phase change is dependent on the acyl chain composition of the cholesteryl ester and on the triacylglycerol/cholesterol ester ratio. It is thus not clear whether cholesterol in the LDL particles may be in an ordered phase in whole or in part. A cryo-electron microscopy study of the LDL ultrastructure

shows an extensive phase-separated ordered state lamellar organization of the lipid core, but this may have ensued from imaging at low temperature (95).

The storage site of cholesterol in the cells is in lipid droplets. Lipid droplets are cellular organelles that are actively engaged in multiple functions. Importantly, they serve as a storage site for cholesterol, which eventually participates in cell membrane structure and maintenance. Lipid droplets consist of an outer lipid monolayer of phospholipids, associated proteins, and cholesterol and a hydrophobic core that consists largely of triacylglycerols and cholesterol esters (**Figure 5**) (96). Some phospholipids and free cholesterol may also exist in the core (93).

Lipid droplets vary greatly in size, ranging from 20–40 nm to 100 μm. In adipocytes (fat cells), lipid droplets tend to be larger and may compose the majority of the cell, while in other cells they may be induced only under certain conditions and are considerably smaller in size. Under normal culture conditions, lipid droplets are not structured; however, in certain cell-cycle stages or metabolic states that may be related to stress responses, they may transition into a smectic liquid-crystalline phase surrounding an amorphous core (97). Thus, lipid droplets can assume different morphologies and structures related to specific cellular conditions, which results in different modes of interaction with cellular organelles. In addition, lipid droplets are a major component of atherosclerotic lesions, and their structural characteristics may play a significant role in lesion development and stability.

Under physiological conditions, cellular trafficking of cholesterol follows a cascade of events consisting of hydrolysis of LDL cholesteryl ester in cellular secondary lysosomal compartments followed by cholesterol trafficking to the plasma membrane and storage in lipid droplets, from which it can be taken up for various functions. When the delicate balance between cholesterol absorption, synthesis, and excretion is broken, and the overall content of free cholesterol rises, pathology-related phenomena involving deposition of crystalline cholesterol ensue. In atherosclerosis, altered LDL particles undergo phagocytosis by macrophage cells, specialized scavengers of foreign invaders in the body. Macrophages process the altered LDL particles, accumulating excess cholesterol in lipid droplets until the macrophages transform into swollen cells called foam cells. Foam cells are characterized by an aberrant accumulation of cytosolic lipid droplets and are considered a hallmark of atherosclerotic lesions through all stages of development. Eventually, crystalline cholesterol appears associated with foam cells, ultimately giving rise to the classic atherosclerotic plaque (98, 99).

Several pathways for cholesterol crystallization in association with macrophages have been proposed involving secondary lysosomal compartments and the cellular plasma membrane (100, 101). In both these instances, cholesterol crystals could nucleate from segregated cholesterol domains in the membranes with the same mechanism that was demonstrated in vitro from macrophage cells or from model mixed lipid bilayers. Varsano et al. (33, 102) demonstrated that triclinic rhomboid plates of cholesterol monohydrate with nanoscopic sizes could nucleate on the plasma membrane of macrophage foam cells. However, monoclinic cholesterol monohydrate crystals with rod-like, tubular, or helical morphology formed intracellularly (33). A recent hypothesis suggests a mechanism whereby monoclinic cholesterol monohydrate crystals may nucleate through epitaxial growth from cholesterol ester ordered domains in lipid droplets (23). Any of these mechanisms might conceivably operate inside a live cell, a dead cell, or the extracellular matrix following cholesterol ester hydrolysis. Cholesterol crystals in atherosclerotic plaques assume both plate and rod or needle morphology (**Figure *6a,b***) (47, 103). The only explanation that has so far been advanced to explain the rod morphology maintains that crystals form in the monoclinic polymorph and preserve the morphology after a single-crystal-to-single-crystal transition to the stable triclinic polymorph (20). This hypothesis, if confirmed, would provide relevant information about the mechanisms of crystal formation in atherosclerosis.

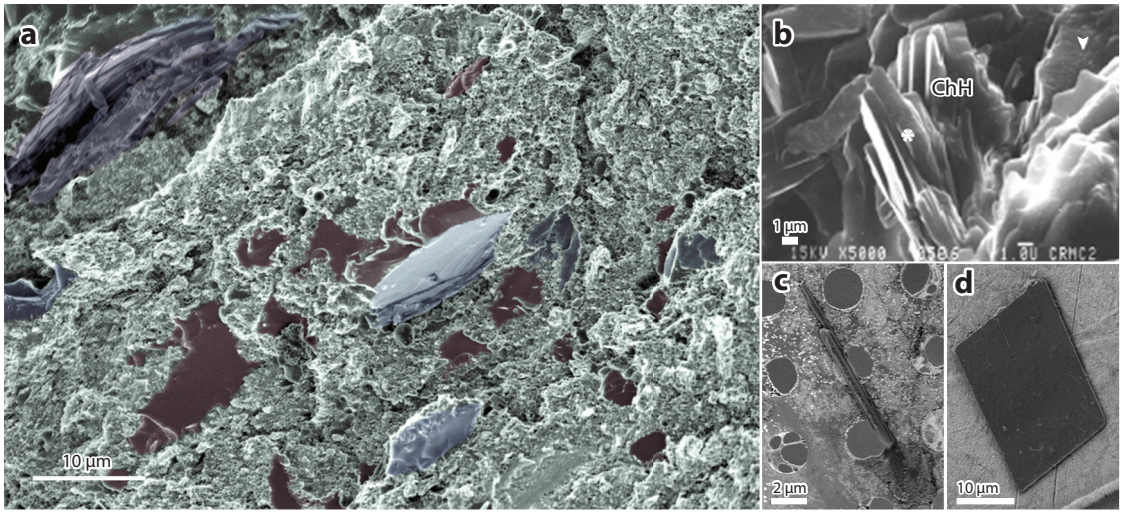

Figure 6

(*a*) Cryo-scanning electron microscopy (SEM) micrograph from a freeze-fractured human atherosclerotic plaque. The cholesterol crystals are colored purple. (*b*) SEM micrograph of cholesterol crystals from a gallstone. The asterisk indicates cholesterol monohydrate (ChH) crystals, and the arrowhead indicates pigment granules. Panel *b* reproduced with permission from Reference 112. (*c*,*d*) Cholesterol crystals extracted from a human atherosclerotic plaque. The crystals are (*c*) rods or (*d*) rhomboid plates.

In addition to cholesterol crystals, most atherosclerotic plaques develop calcifications of apatite (calcium phosphate) crystals. In 1968, Kathleen Lonsdale published a historical paper (104) in which she pointed out the possibilities of epitaxial matches and, consequently, epitaxial growth of different crystals in gallstones, including apatite. Swift and colleagues (105) later widened and formalized the search for epitaxial matches, and it is fascinating to think that this may also apply to atherosclerosis.

Ultimately, crystalline cholesterol is very difficult to solubilize using known physiological pathways after its formation, securing its presence for years to come and perpetuating the process of lesion growth (86). When crystallization of cholesterol occurs within an atherosclerotic lesion, cholesterol crystals lead to traumatic injuries to the diseased tissue (106). Direct proinflammatory effects are also associated with cholesterol crystals (107, 108), which, coupled with repetitive cyclic stresses that weaken the structures and increase the disposition of the plaque to fracture, may lead to mechanical failure caused by fatigue. Inflammation and direct mechanical damage induced by the crystals weaken the fibrous cap, which may ultimately rupture unprompted (109, 110), pouring into the blood masses of crystals and cellular and extracellular matrix debris that may cause blood vessel obstruction, leading to heart attack and stroke.

A study of human aortic atherosclerotic plaques reports that the transition from intact to disrupted lesions is accompanied by an increase in cholesterol, cholesteryl esters, and the percentage of unesterified cholesterol relative to esterified cholesterol in the necrotic core, whereas the triglyceride content remains unchanged. The influence of lipid composition on plaque instability in coronary sudden death is also apparent from data showing that the percentage of cholesterol crystals is greater in ruptured lesions than in eroded or stable plaques (111).

Cholesterol crystallization from saturated bile is another pathology that may result from disruption of cholesterol homeostasis. Bile is the major route for elimination of cholesterol from the body through solubilization by bile acids in the gall bladder followed by secretion of cholesterol into the intestinal tract.

Bile is a fluid synthesized in the liver. It consists of ~95% water and ~5% dissolved organic molecules, predominantly bile acid salts, phospholipids, and cholesterol. In physiological conditions, biliary cholesterol dissolves within salt-rich micelles and phospholipid-rich vesicles (113). Supersaturation of cholesterol in bile may prompt the crystallization of cholesterol from these metastable lipid aggregates within the gallbladder, subsequently forming gallstones. Multilamellar vesicles in bile pave the way for and might even have a direct role in the initiation of crystallization of cholesterol in gallbladder bile (114, 115). In a model system containing mixed bile salts, lecithin, and cholesterol, as well as in human bile, distinct crystal morphologies were observed to form, comprising liquid crystals, filamentous crystals, curved crystals, helices, and tubes, aside from stable rhomboid cholesterol monohydrate crystals. These may well correspond to the two polymorphs of cholesterol monohydrate, with the metastable monoclinic polymorph transforming with time into the stable triclinic polymorph. Interestingly, the formation of distinct crystalline cholesterol structures in bile depends on the ratios of its lipid constituents. Differences in model bile lipid composition give rise to substantially different kinetics of the transformation between the metastable helices and the stable rhomboid plates, indicating different pathways of crystal nucleation and growth leading to the thermodynamically stable polymorph (115).

In an interesting case study that was not aimed at establishing crystal structures at all, Landi et al. (35) examined bile from patients with a clinical diagnosis of acalculous gallbladder disease and compared it to bile from patients with known gallstones. They reported the presence of predominantly needle-like crystals in most patients without gallstones and of plate-like crystals in patients with gallstones. If indeed the two groups correspond to the two cholesterol monohydrate polymorphs, analysis of the polymorphism of cholesterol could once again provide relevant information on the environments and the mechanisms of cholesterol crystal formation.

CONCLUDING REMARKS

Cholesterol or other sterols are essential components of all eukaryotic cell membranes. Interestingly, cholesterol is unique in animal cell membranes in the sense that membrane composition includes thousands of lipids and thousands of proteins but only one sterol, cholesterol. Why is cholesterol so unique? It is common knowledge that cholesterol is indispensable for membrane integrity and function, because it controls lipid order, fluidity, permeability, and mechanical coherence (116). It is also well known that excess cholesterol may result in pathologies and that strokes or heart attacks are common and often lethal consequences of cholesterol accumulation in the arteries.

Cholesterol is also an essential component of carriers used for drug delivery. As an example, cholesterol is a major constituent of lipid nanoparticles used for small interfering RNA delivery, where it contributes to morphology, stability, fluidity control, and successful delivery to the target organs (117, 118). Lipid nanoparticles are but a small fraction of the vast and growing interest in cholesterol and cholesterol-based compounds for applications in drug delivery, such as use of bile acids as absorption enhancers for drugs (119).

In all these instances, what is unique to cholesterol are the material properties that derive from its unique molecular configuration and consequently from its unique modes of assembly. Cholesterol molecules can be considered as free but rigid rotators in monolayers when alone or mixed with other lipids, such as in the envelopes of the lipid–messenger RNA complexes forming lipid nanoparticles (117, 118). Presumably, the same occurs at the surface of lipid droplets, which are covered by a single layer of cholesterol and phospholipids, and in mixed ordered domains in cell membranes, which have monolayer thickness. In fact, we suspect that the composition range for formation of such ordered nanoscopic domains with saturated glycerophospholipids or sphingolipids is much wider than what conventional phase diagrams imply.

When its concentration is higher than the solubility in the lipid environment of lipid bilayer membranes, cholesterol may associate with itself, forming crystalline bilayer nanodomains, which likely influence the mechanical properties of the membrane because of their rigidity and structure. When the whole environment is supersaturated in cholesterol, the membrane domains may serve as templates for the nucleation of crystals.

Interestingly, cholesterol may form at least two monohydrate polymorphic crystal structures with very different properties from membranes in aqueous environments. The metastable monoclinic polymorph favors extremely thin crystals, which may assume helical morphologies when crystallized from bile but also from mixed bilayers with saturated glycerophospholipids. Because of their morphology and lower stability, the monoclinic crystals are expected to have higher solubility and different material properties than the far more common triclinic crystals. The monoclinic polymorph may also conceivably be a precursor of the triclinic one in pathological settings. If this is true, its different material properties could be exploited to solubilize the crystals or to avoid atherosclerotic plaque rupture if the transformation into the stable polymorph could be slowed down.

In conclusion, the material properties of cholesterol in its various aggregation states are central to its behavior, both in physiology and in pathology.

DISCLOSURE STATEMENT

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